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Molecular Immunology, Vol. 26, No. 12, pp. 1155–1167, 1989
Printed in Great Britain.

0161-5890/89 $3.00 + 0.00
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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTI-CD18 ANTI-IDIOTYPE ANTIBODIES*

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(*First received* 13 *January* 1989; *accepted* 5 *June* 1989)

Abstract—Three leukocyte adhesion receptors have been described which mediate intercellular binding of leukocytes: LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and p150/95 (CD11c/CD18). We have previously reported the production of several monoclonal antibodies against the common subunit of these receptors (CD18). We here describe the production of monoclonal anti-idiotype antibodies against one of the anti-CD18 antibodies (H52) which has been shown to inhibit potently the function of leukocyte adhesion receptors. Three IgG1 and two IgM anti-idiotype antibodies were derived which recognized private idiotopes on the H52 molecule. Two of these antibodies blocked the binding of H52 to purified LFA-1 and to cell surface expressed antigen. One of the antibodies (AIM.6) was shown to be an internal image-type (Ab2β) antibody based on inhibition of its binding to H52 by purified LFA-1 and by its ability to induce Ab3 which recognize LFA-1 when used as immunogen. The AIM.6 Ab2β antibody was tested for recognition of leukocyte adhesion ligands in LFA-1-mediated leukocyte adhesion and activation assays. The AIM.6 antibody did not block intercellular adhesion of leukocytes or mitogen stimulation of T cells, functions which were completely inhibited by low concns of H52. AIM.6 Ab2β antibody bound to H52 very well at 0°C but bound very poorly or not at all at 37°C. Binding studies on a panel of anti-CD18 monoclonal antibodies showed that the idiotope defined by AIM.6 was unique to H52 and an antibody recognizing the same epitope on CD18 (H5B9). This result showed that inhibitory anti-CD18 monoclonal antibodies utilize at least two distinct paratopes in binding to CD18. The above results are in contrast to those obtained in other systems in which Ab2β antibodies against receptor-specific Ab1 antibodies recognize receptor ligands and are discussed in the context of ligand recognition by leukocyte adhesion receptors.

INTRODUCTION

LFA-1, Mac-1, and p150/95 molecules constitute an important family of leukocyte adhesion receptors (LAR) (Springer *et al.*, 1987). The LFA-1 molecule consists of an α-subunit of M_r 180,000 (CD11a) associated with a β-subunit of M_r 95,000 (CD18) (Sanchez-Madrid *et al.*, 1983*a*; Hildreth and August, 1985). The LFA-1 molecule is widely expressed on cells of hematopoietic lineage and is involved in both antigen-dependent and antigen-independent lymphocyte adhesion (Springer *et al.*, 1987; Campana *et al.*, 1986; Kurzinger *et al.*, 1981). The Mac-1 and p150,95 molecules consist of α-subunits of M_r 165,000 (CD11b) and 150,000 (CD11c), respectively, associated with a β-subunit (CD18) identical to that of LFA-1 (Hildreth and August, 1985; Sanchez-Madrid *et al.*, 1983*a*; Springer *et al.*, 1986; Lanier *et al.*, 1985). Mac-1 and p150,95 have a more restricted tissue expression, being limited to granulocytes and macrophages (Hogg *et al.*, 1986; Springer *et al.*, 1982).

Monoclonal antibodies (mAb) against LFA-1 have been shown to inhibit a wide variety of leukocyte adhesion functions. Antibodies against LFA-1 inhibit mixed lymphocyte reactions, antigenic and mitogenic T cell stimulation, cytotoxic T cell and natural killer cell function, as well as antibody-dependent cell-mediated cytotoxicity (Springer *et al.*, 1982; Springer *et al.*, 1987; Hildreth *et al.*, 1983; Hildreth and August, 1985) by blocking the required cell–cell interactions. T cell-dependent antibody responses by B cells are also inhibited by anti-LFA-1 antibodies, presumbly as a result of blockade of B cell interactions with T helper cells or antigen presenting cells (Fischer *et al.*, 1986*b*; Howard *et al.*, 1986). The Mac-1 molecule has been identified as the type three

*This work was supported by grants from the National Science Foundation (DCB-8657488-104), Johns Hopkins University School of Medicine (Johns J. Hopkins Fund), Johnson and Johnson, and the Andrew W. Mellon Foundation. J. E. K. Hildreth is the recipient of an NSF Presidential Young Investigator Award.

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Abbreviations: Ab1, primary antibody; Ab2, anti-idiotype antibody; Ab3, anti-Ab2 antibody; Ab2β, β-type anti-idiotype antibody; CR3, type-3 complement receptor; ECMR, extracellular matrix receptor; FBS, fetal bovine serum; ICAM-1, intercellular adhesion molecule-1; KLH, keyhole limpet hemocyanin; LAR, leukocyte adhesion receptor; mAb, monoclonal antibody(ies); PVC, polyvinyl chloride; RAMIg, rabbit anti-mouse immunoglobulin (μ-, γ-, and L-specific); RAMγ, rabbit anti-mouse immunoglobulin (γ Fc-specific); RAMμ, rabbit anti-mouse immunoglobulin (μ Fc-specific); RGD, Arg–Gly–Asp tripeptide; SPRIA, solid phase radioimmunoassay; TPA, tetradecanoyl phorbol-13-acetate.

complement receptor (CR3) and appears to serve as a general adhesion molecule for granulocytes and macrophages (Beller *et al.*, 1982; Hildreth and August, 1985; Sanchez-Madrid *et al.*, 1983*b*; Anderson *et al.*, 1986; Wright *et al.*, 1983; Patarroyo *et al.*, 1985*b*). CR3 activity has also been demonstrated for the p150,95 leukocyte adhesion receptor (LAR) (Miller *et al.*, 1987). MAb against Mac-1 have been shown to inhibit CR3 activity and recent studies indicate that anti-Mac-1 mAb also inhibit granulocyte adhesion *in vitro* and *in vivo* (Anderson *et al.*, 1986; Wallis *et al.*, 1985; Simpson *et al.*, 1988). In all of the above *in vitro* studies in which leukocytes are coated with mAb against LAR, the functional defects produced appear to mimic those observed in a life-threatening immunodeficiency disease in which expression of LAR is moderately to severely deficient (Springer *et al.*, 1984). The broad immunosuppression produced by anti-LFA-1 mAb *in vitro* have now been successfully produced *in vivo* in the treatment of allograft rejection (Fischer *et al.*, 1986*a*). The results of *in vitro* functional studies, clinical manifestations of LAR deficiency, and the dramatic success of anti-LFA-1 treatment of bone marrow transplants clearly demonstrate the importance of LAR in the functioning of the immune system.

Primary characterization of the subunits of LAR show that these molecules belong to a large family of adhesion molecules known as integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). The integrin glycoprotein family includes receptors for extracellular matrix molecules (ECMR) such as fibronectin, vitronectin, fibrinogen, and von Willebrand protein (Ruoslahti and Pierschbacher, 1987). Integrins appear to play important roles in cell migration and embryogenesis, cell differentiation and morphology, and other processes such as wound healing, thrombosis, hemostasis, malignant transformation and immume activation (Hynes, 1987). Like LAR other integrins consist of two subunits, α and β, in a 1:1 non-covalent complex. Both subunits are transmembrane glycoproteins and interact with ligands via large extracellular domains and with the cytoskeleton via relatively small intracellular domains. Primary characterization of integrins indicate that there are at least three different β-subunits and 10 different α-subunits (DeSimone and Hynes, 1988). There are currently three known sub-groups within the integrin family in which a set of different α-subunits associate with one of the three β-subunits (Hynes, 1987; Hemler, 1988).

The ECMR integrins have been shown to recognize a core amino acid sequence of arginine-glycine-aspartate (RGD) in the ligand polypeptides (Ruoslahti and Pierschbacher, 1987). There is evidence that the binding of C3bi, one of the ligands of Mac-1 and p150,95, also involves an RGD sequence (Wright *et al.*, 1987). At present there is no evidence that binding of LFA-1 involves this tripeptide sequence. Cell adhesion and aggregation studies indicate that there are at least two distinct ligands for LFA-1 (Rothlein and Springer, 1986; Dustin and Springer, 1988). MAb have made possible the characterization of one of the LFA-1 ligands, the intercellular adhesion molecule-1 (ICAM-1) (Rothlein *et al.*, 1986; Dustin *et al.*, 1986). Elucidation of the primary structure of ICAM-1 has shown it contains no RGD sequences and is a member of the immunoglobulin supergene family (Staunton *et al.*, 1988). ICAM-1 is expressed widely on hematopoietic and non-hematopoietic tissue and its density on fibroblasts and endothelial cells is increased substantially by soluble mediators such as IL-1 and IFN-γ (Dustin *et al.*, 1986). MAb against ICAM-1 inhibit LFA-1-mediated homotypic lymphocyte aggregation and also adhesion of lymphocytes to fibroblasts and endothelial cells (Dustin and Springer, 1988). In these studies mAb against ICAM-1 inhibited most but not all LFA-1-mediated interactions reflecting the existence of other potential ligands. As discussed above, Mac-1 and p150/95 are known to bind to C3bi but Mac-1 also appears to mediate granulocyte adhesion to endothelial cells. The ligands on endothelial cells responsible for this Mac-1-mediated interaction have not been identified.

Anti-idiotype antibodies have emerged as useful tools for identifying receptors and their ligands. These studies are based on Jerne's network hypothesis (Jerne, 1974) in which the immune system is envisaged as an interacting network of idiotypes and anti-idiotypes. This model predicts that for every exogenous immunogenic epitope encountered by the immune system there exists an anti-idiotype antibody carrying an "internal image" of this epitope in its hypervariable region. Such anti-idiotype antibodies have been designated antibody 2 beta (Ab2β) (Bona and Kohler, 1984). When antibodies produced against the binding site of a receptor (Ab1) are used as immunogens the idiotype network hypothesis predicts that anti-idiotype antibodies of the Ab2β type will behave as receptors and recognize the ligand(s) of the receptor. Several receptor systems have been described in which anti-idiotypic antibodies have been shown to block or mimic the binding of the physiological ligand to its receptor. These include prolactin, thyroid stimulating hormone, retinol-binding protein, insulin, β-adrenergic and acetylcholine receptors (Monroe and Greene, 1986). Anti-idiotype antibodies have also been used to identify the cell receptors for virus envelope glycoproteins, including reovirus, murine leukemogenic retroviruses, human immunodeficiency virus and Epstein–Barr virus (Kauffman *et al.*, 1983; Chanh *et al.*, 1987; Hiernaux, 1988; Barel *et al.*, 1988).

We have begun a series of experiments in which anti-idiotype antibodies against anti-LAR mAb will be tested for recognition of leukocyte adhesion ligands. Here we report the production and characterization of monoclonal anti-idiotype antibodies against H52, an anti-LFA-1 β-subunit (CD18) mAb which

potently inhibits LFA-1-mediated adhesion. One such anti-idiotype mAb (AIM.6) was confirmed to be of the Ab2β type but did not appear to recognize an LFA-1 ligand based on cell adhesion and antibody binding studies. The AIM.6 idiotope was expressed on only two of 12 anti-CD18 mAb, at least six of which are known to inhibit LFA-1 function, suggesting that multiple anti-CD18 idiotopes are associated with inhibition of LFA-1 function.

MATERIALS AND METHODS

Animals

Balb/c and CB6F1 female mice (6–8 weeks old) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and maintained in the animal facility of the Department of Pharmacology and Molecular Sciences.

Cells

Human cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 10 m*M* HEPES, and 2 m*M* L-glutamine (complete medium). The following cell lines were used: U-937, myelomonocytic leukemia; SW.B, EBV transformed B cell; HSB-2, T cell leukemia; and K-562, erythroleukemia. Human peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque density centrifugation (Boyum, 1968). Neutrophils were isolated by hypotonic lysis of red cells in the Ficoll-Hypaque pellets. Phytohemagglutinin (PHA) blasts were generated by incubating PBMC at a density of 2×10^6/ml in complete medium in the presence of 0.5 μg/ml PHA (Wellcome Diagnostics, Triangle Park, NC) for 3 days at 37°C in a humidified 5% CO_2 incubator. Hybridoma cells and the P3x653.Ag8 myeloma line were maintained in DMEM supplemented with 10% FBS, 10% NCTC-135, 10 m*M* HEPES, 0.2 U/ml insulin, 0.45 m*M* pyruvate, and 1 m*M* oxaloacetate. Hybridoma cells were fixed with paraformaldehyde as follows. Cells were washed three times with PBS and resuspended in 2% paraformaldehyde in PBS at 10^7/ml for 10 min at room temp. After washing three times with 50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, the cells were resuspended in PBS and stored at −70°C.

Monoclonal antibodies

The production and characterization of some of the mAb used in this study have been reported (Hildreth and August, 1985; Dongworth *et al.*, 1985; Hildreth *et al.*, 1983). H52, H5B9, and MHM.23 are all IgG1,k isotype and recognize the β-subunit of LFA-1 (CD18). MHM.24, IgG1,k recognizes the α-subunit of LFA-1 (CD11a). PLM.19, PLM.22, PLM.31, and PLM.34 were produced by immunizing Balb/c mice with affinity purified porcine LFA-1 and all recognize epitopes on human and porcine CD18. Other CD18-specific mAb were obtained from the Third International Leukocyte Workshop.

Purification of LFA-1

LFA-1 was purified from human and porcine spleen by affinity chromatography on an anti-CD18 (MHM.23) column exactly as previously described (Hildreth and August, 1985). Human CD45 (leukocyte common antigen) was similarly purified from human spleen by affinity chromatography. Purity of the isolated proteins was confirmed by SDS–PAGE (Laemmli, 1970).

Antibody purification and production of F(ab')$_2$ fragments

H52 IgG was purified from ascites fluid by HPLC ion exchange chromatography as previously described (Deschamps *et al.*, 1985) except that a 45% ammonium sulfate fractionation was carried out prior to the HPLC separation. IgM mAb were purified from ascites fluid by 35% ammonium sulfate fractionation followed by dialysis of the resuspended pellet against 5 m*M* Tris–HCl, pH 8.0. The precipitated IgM was taken up in PBS and adjusted to 2 mg/ml. F(ab')$_2$ fragments were produced by a modification of a previously described method (Parham, 1983). Ascites fluids were dialyzed overnight against 0.1 *M* sodium citrate, pH 3.5. Pepsin (Sigma Chemical Co., St Louis, MO) was then added to a final concn of 100 μg/ml and the samples were incubated for 12 to 15 hr at 37°C. After dialysis against 20 m*M* Tris–HCl, pH 8.5, the F(ab')$_2$ fragments were isolated by ion exchange HPLC as previously described for IgG (Deschamps *et al.*, 1985). Purity of all IgG, IgM, and F(ab')$_2$ preparations was confirmed by SDS–PAGE. F(ab')$_2$ preparations did not react with rabbit antibody against the Fc fragment of mouse IgG (RAMγ).

Antibody conjugation and radioiodination

Purified antibodies were conjugated to keyhole limpet hemocyanin (KLH) as follows. Equal volumes of KLH and antibody each at 2 mg/ml in PBS were mixed and glutaraldehyde was added for a final concn of 0.08%. After mixing end-over-end for 1 hr the samples were dialyzed exhaustively against PBS. Purified antibodies were labeled with ^{125}I using insoluble lactoperoxidase and glucose oxidase (EnzymoBeads, BioRad Laboratories, Richmond, CA). Briefly, 20 μg of purified antibody was mixed with 50 μl of EnzymoBeads in 0.2 *M* sodium phosphate, pH 7.2. After adding 0.5 mCi of ^{125}I the mixture was incubated for 30 min at 25°C before desalting the labeled protein on a 10-ml Sephadex G-25 medium column equilibrated with PBS. Affinity purified rabbit anti-mouse γ-chain (RAMγ), rabbit anti-mouse μ-chain (RAMμ), and rabbit anti-mouse $\gamma + \mu + L$ chains (RAMIg) were all obtained from Jackson ImmunoResearch (West Grove, PA) and were radioiodinated using chloramine T as previously described (Greenwood *et al.*, 1963).

Immunization and fusions

Female Balb/c mice (6 weeks old) were injected i.p. with 10^7 paraformaldehyde-fixed hybridoma cells and 100 μg of mAb–KLH conjugate in CFA. After 7 days the mice received 50 μg of mAb–KLH conjugate i.p. in PBS. One week later 50 μg of conjugate in ICFA were injected i.p. Then at 2 week intervals the mice received 50 μg of conjugate i.v. in PBS. Four days after the final i.v. injections spleens were removed for fusions. Spleen cells were fused with P3x653.Ag8 myeloma cells as previously described using 50% polyethylene glycol (ATCC, Rockville, MD) (Hildreth *et al.*, 1983; Bastin *et al.*, 1982). Fused spleen cells from a single immunized mouse were seeded into eight flat-bottom 96-well plates (Costar, Cambridge, MA) using spleen cells from two normal CB6F1 mice as feeder cells. Fusions were screened by solid phase radioimmunoassays (SPRIA) as described below. Selected hybridomas secreting antibodies of interest were cloned twice by limiting dilution as previously described (Hildreth *et al.*, 1983; Bastin *et al.*, 1982). Ascites fluid was prepared by injecting 10^7 hybridoma cells into the peritoneal cavities of CB6F1 female mice 7 days after an i.p. injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane (Aldrich Chemical Co., Milwaukee, WI). MAb were isotyped by ELISA using a kit from Zymed Laboratories (South San Francisco, CA) according to the manufacturer's protocol.

Solid phase radioimmunoassays (SPRIA)

$F(ab')_2$ fragments were diluted to 10 μg/ml in 50 m*M* Tris, pH 9.5, and 50 μl of the diluted protein were added to the wells of polyvinyl chloride (PVC) microtiter plates (Costar, Cambridge, MA). The plates were incubated overnight at room temp or placed under the air stream of a hair dryer set at low speed and temp for 45 min. The wells were then blocked with 100 μl/well of 3% BSA/PBS for 45 min at 4°C. After washing five times with PBS, 0.1% BSA, 0.05% Tween 20 (PBT buffer), 50 μl of hybridoma culture supernatants from fusion wells or diluted ascites were added to duplicate wells and the plates incubated for 1 hr at 4°C. The wells were then washed five times with PBT buffer followed by the addition of 50 μl of diluted radiolabeled second antibody (4–6 × 10^5 cpm/well), RAMμ or RAMγ. After a 1-hr incubation at 4°C the wells were washed five times with PBT, separated and assayed for bound radiolabel. For determining the frequency of occurrence of the AIM.6-defined idiotope, purified RAMγ was dried down in PVC wells and blocked as described above. Various CD18-specific antibodies diluted in PBS, 0.5% BSA were then added to the wells followed by a 1-hr incubation at 4°C. After washing five times with PBT buffer, radiolabeled AIM.6 or RAMIg was then added to the wells followed by a 1-hr incubation at 4°C. The wells were washed five times with PBT buffer, separated, and assayed for bound radioisotope.

Competitive binding studies

Competitive binding studies were carried out using two methods. In the first, diluted ascites fluids or culture supernatant from anti-idiotype hybridomas were mixed with an equal volume (75 μl) of a 1:100 dilution of H52 culture supernatant and incubated for 1 hr on ice. Fifty μl of the mixture were then added in duplicate to 5 × 10^5 HSB-2 cells in 50 μl of PBS, 0.5% BSA, 0.2% sodium azide (PBA buffer). After a 1-hr incubation on ice the cells were washed twice with PBS, 0.2% sodium azide, 0.1% BSA (PAB buffer). Radiolabeled RAMγ (500,000 cpm) was then added followed by a 1-hr incubation on ice. The cells were washed twice with PAB buffer and assayed for bound radiolabeled antibody. In the second method radiolabeled mAb (10 μCi/μg) was mixed with an equal volume of culture supernatants or diluted ascites fluids and incubated for 45 min on ice. The mixtures were then added to HSB-2 cells or the wells of PVC plates coated with purified $F(ab')_2$ fragments or affinity purified LFA-1. After a 1-hr incubation at 4°C HSB-2 cells were washed twice with PAB buffer; plates were washed four times with PBT buffer.

Cell surface antibody binding studies

Binding of mAb to cell surface structures was determined as described (Makgoba *et al.*, 1983). Briefly, 50 μl of cells at a density of 10^7/ml in PBA buffer were mixed with an equal volume of mAb (culture supernatant or diluted ascites fluid) and incubated for 1 hr at 4°C. After two washes with PAB, radioiodinated (approx 500,000 cpm) RAMγ or RAMμ was added followed by a second 1-hr incubation at 4°C. The cells were then washed twice with PAB buffer and assayed for bound radiolabel.

Cell aggregation assay

Cell aggregation assays were performed essentially as previously described (Rothlein and Springer, 1986; Patarroyo *et al.*, 1985*a*). Briefly, washed cells in complete medium at a density of 2 × 10^6/ml were mixed with an equal volume (50 μl) of inhibiting antibody in 96-well round-bottom plates (Costar, Cambridge, MA) and incubated for 20 min at room temp. Fifty μl of complete medium containing 150 ng/ml of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA; Sigma Chemical Co.) were then added to the wells followed by incubation at 37°C for 1 hr. Negative control wells received 50 μl of complete medium without TPA. The cells were gently resuspended and counted in a hemacytometer. Per cent aggregation was determined by the following formula:

$$\frac{\text{single cell count without TPA} - \text{single cell count with TPA}}{\text{single cell count without TPA}} \times 100.$$

Assays were carried out in duplicate and repeated at least twice with similar results.

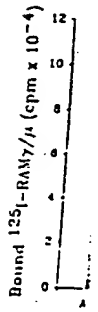

Fig. 1. Spec
supernatant
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Mitogen stimulation assays

Inhibition of PHA stimulation of T cells by mAb was carried out as described previously (Hildreth and August, 1985). Briefly, an equal volume (50 μl) of PBMC suspension (4×10^6/ml) and purified mAb each in complete medium were mixed and incubated for 20 min at room temp. PHA was then added to final concns of 1, 0.5, 0.25 and 0.125 μg/ml followed by incubation for 3 days at 37°C in a humidified CO_2. One μCi of ^{3}H-thymidine was added to each well for the final 18 hr of culture. Cells were harvested onto glass fiber filter discs and assayed for incorporated isotope.

Cell labeling and immunoprecipitation

Vectorial cell surface labeling of HSB-2 cells, detergent lysis, and immunoprecipitation with polyclonal sera and mAb were carried out exactly as previously described (Hildreth and August, 1985).

RESULTS

Production and selection of anti-CD18 anti-idiotype mAb

Balb/c mice were immunized with purified H52 Ig coupled to KLH and paraformaldehyde-fixed H52 hybridoma cells as described in Materials and Methods. A solid phase radioimmunoassay (SPRIA) was developed to screen for anti-idiotype antibodies. Sera from immunized mice were tested against H52 $F(ab')_2$ fragments in SPRIA employing radioiodinated RAMγ or RAMμ as second antibody. Sera from all of the immunized mice showed anti-H52 antibody activity at dilutions greater than 3000^{-1} (data not shown). Since the H52 cell line and mAb are syngeneic to Balb/c mice all of the observed anti-H52 antibody activity presumably represented anti-idiotype antibodies. No binding was seen with normal mouse serum demonstrating the absence of detectable whole IgG in the H52 $F(ab')_2$ preparation.

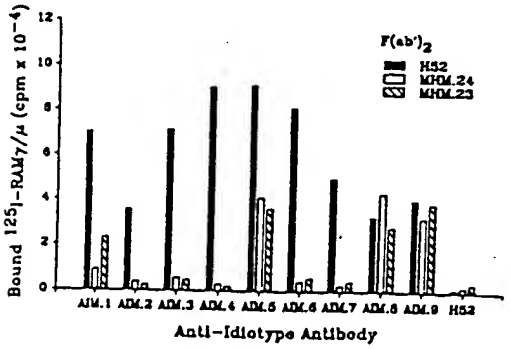

Fig. 1. Specificity of anti-H52 anti-idiotype mAb. Culture supernatants from Ab2 secreting hybridomas were tested against three different Ab1 $F(ab')_2$ in SPRIA using RAMγ and RAMμ as second antibody. Ab1 $F(ab')_2$ used were (all IgG1,k): H52, anti-CD18; MHM.23, anti-CD18; and MHM.24, anti-LFA-1 α-subunit (CD11a).

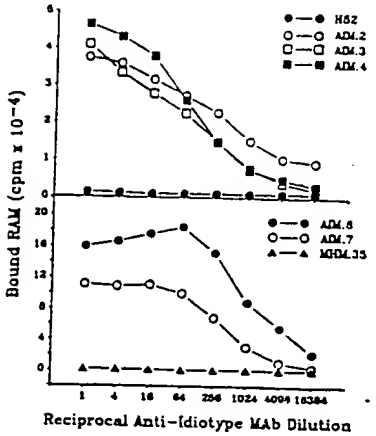

Fig. 2. Titration of anti-idiotype mAb binding to H52 $F(ab')_2$. Culture supernatants from Ab2 secreting hybridomas and H52 cells were diluted in PBA buffer as shown and tested for binding to H52 $F(ab')_2$ in SPRIA as detailed in Materials and Methods. Upper panel: IgG1,k Ab2 mAb were tested using RAMγ (500,000 cpm/well) as second antibody. Lower panel: IgM,k Ab2 were tested using RAMμ (500,000 cpm/well) as second antibody. MHM.35, an IgM,k mAb against HLA-DR, was used as a negative control.

Nine Ab2, designated AIM.1 to AIM.9, were selected for further characterization based on antibody binding to H52 $F(ab')_2$ in SPRIA. AIM.2, AIM.3, and AIM.4 are IgG1,k; the other Ab2 mAb are IgM,k. The specificities of the nine Ab2 mAb were determined by SPRIA using $F(ab')_2$ fragments of three different Ab1 as target antigen. Five of the nine mAb (AIM.2, AIM.3, AIM.4, AIM.6 and AIM.7) recognized a determinant on H52 $F(ab')_2$ but not on the two control Ab1 $F(ab')_2$, MHM.23 which recognizes a second epitope on CD18, and MHM.24 which is specific for the LFA-1 α-subunit (Fig. 1). These five H52-specific mAb presumably react with private idiotopes on H52. The other anti-idiotype antibodies appeared to react with public or shared idiotopes as they reacted to varying degrees with all three Ab1 $F(ab')_2$. Since all three Ab1 $F(ab')_2$ are IgG1,k isotype, the cross-reactive mAb antibodies may recognize a framework determinant on the G1 heavy chain or a determinant on the κ light chain. The specificities of the cross-reactive Ab2 were not further determined. H52 whole IgG was used as a negative control and showed no binding to any of the $F(ab')_2$ preparations. The anti-idiotype mAb activity of culture supernatants from hybridomas secreting Ab2 against private idiotopes was titrated in SPRIA against H52 $F(ab')_2$. The IgG1 Ab2 (AIM.2, AIM.3 and AIM.4) all bound equally well to H52 $F(ab')_2$ but, despite starting antibody concns of greater than 10 μg/ml in the culture supernatants, plateau binding was not seen with any of these Ab2 (Fig. 2). No binding was seen with H52 supernatant which was used as a negative control. The H52-specific IgM Ab2 (AIM.6 and AIM.7) also bound well to H52 $F(ab')_2$ and appeared to bind with a much higher affinity than the IgG1 Ab2 (Fig. 2). Both AIM.6 and AIM.7 were

determined to be pentameric IgM by size exclusion HPLC and SDS–PAGE. Thus the higher binding titers of these mAb may reflect the higher avidity characteristic of pentameric IgM antibodies. There was no binding of the control IgM mAb MHM.35 which is specific for HLA-DR.

Inhibition of H52 binding by Ab2

The nine anti-idiotype antibodies were tested for inhibition of binding of radioiodinated H52 to LFA-1 expressed on HSB-2 cells and to purified LFA-1 in solid phase. Ascites fluid diluted 1:20 was used for all of the antibodies except AIM.4. Repeated attempts to produce AIM.4 ascites failed, thus this mAb was used as neat hybridoma culture supernatant. Only two of the antibodies (AIM.3 and AIM.6) significantly inhibited the binding of H52 to HSB-2 cells in liquid phase and to purified LFA-1 in solid phase (Fig. 3). AIM.4, AIM.7 and AIM.8 inhibited binding to purified LFA-1 but not to LFA-1 expressed on cells. The inhibition of H52 binding to purified LFA-1 by AIM.8 was probably due to steric effects since this antibody recognizes a public idiotope. This result shows that epitopes on LFA-1 in solid phase may be presented differently than on cell surface expressed antigen which is consistent with observations in other systems (Jemmerson, 1987) and emphasized the importance of using native antigen for inhibition studies. Titration of anti-idiotype mAb ascites fluids against ^{125}I-H52 again showed that only AIM.3 and AIM.6 inhibited binding of H52 to HSB-2 cells (Fig. 4). The inhibition by AIM.3 was relatively weak (50% inhibition titer of 20^{-1}) compared to AIM.6 and unlabeled H52 (50% inhibition titers of 500^{-1} and 6250^{-1}, respectively). Based on titration of antibody activity in the ascites fluids (not shown) the failure of the other antibodies to inhibit H52 binding was not due to lower antibody levels. These results

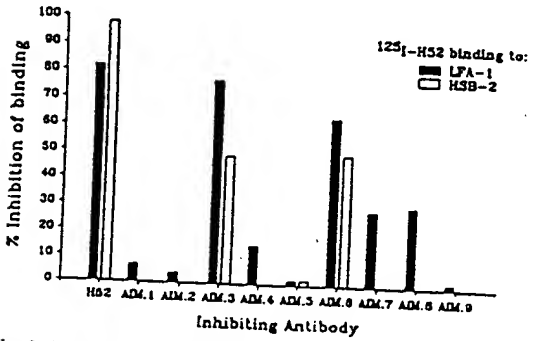

Fig. 3. Inhibition of H52 binding by anti-idiotype mAb. For all mAb except AIM.4, which was used in the form of neat hybridoma culture supernatant, ascites fluids diluted 1:20 in PBA buffer were used. Inhibiting mAb were mixed with an equal volume (75 μl) of radioiodinated H52 (50 ng) and incubated for 1 hr on ice. The mixture was then added in duplicate to purified LFA-1 in PVC plates or to 5×10^5 HSB-2 cells followed by a 1-hr incubation and washing. Maximum binding was determined in the presence of P3x653 culture supernatant.

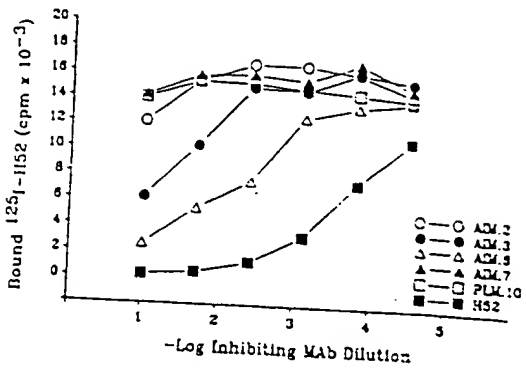

Fig. 4. Titration of inhibition of ^{125}I-H52 binding by anti-idiotype mAb. Serial five-fold dilutions of ascites fluids were prepared starting at 1:10 and tested for inhibition of ^{125}I-H52 binding to HSB-2 cells as described in Fig. 5. PLM.10, an IgG1.k mAb against porcine immunoglobulin, was used as a negative control.

suggested that only AIM.3 and AIM.6 recognized determinants associated with the H52 paratope.

To rule out the possibility that radioiodination of H52 may have altered the antigen recognition site and hence the binding of the anti-idiotype mAb, inhibition studies were also carried out using unlabeled H52. We first confirmed that there was no binding of the H52-specific anti-idiotype mAb to HSB-2 cells (data not shown) and then tested the mAb for blocking activity against unlabeled H52 mAb in an indirect radioimmunoassay. In these assays the anti-idiotype antibodies were mixed with H52 hybridoma supernatant diluted to yield 50% maximal binding (1:100) and after a brief incubation the mixtures were added to HSB-2 cells. Similar to its effect on labeled H52, AIM.6 significantly inhibited binding of unlabeled H52 to HSB-2 cells with a 50% inhibition titer of 1000^{-1} (Fig. 5). In contrast to its low inhibition of radiolabeled H52, AIM.3 strongly inhibited binding of unlabeled H52 to HSB-2 cells (Fig. 5) suggesting that the idiotope on H52 recognized by AIM.3 was

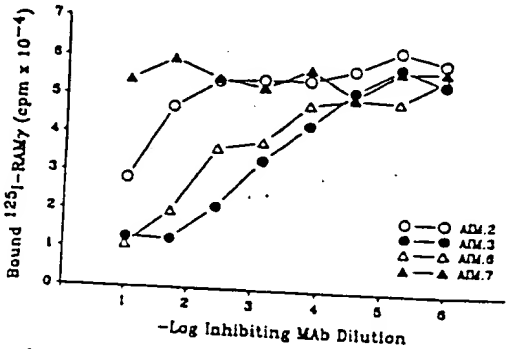

Fig. 5. Titration of inhibition of unlabeled H52 binding by anti-idiotype mAb. Assay was carried out as described in Fig. 6 except that H52 hybridoma supernatant diluted 1:100 in PBA was used instead of ^{125}I-H52 and ^{125}I-RAMγ was used as second antibody. In the absence of added H52 there was no binding of ^{125}I-RAM above control (5300 cpm. P3x653 culture supernatant) for any of the Ab2.

altered by the labeling procedure. AIM.2 ascites also inhibited binding of unlabeled H52 but only at very low dilutions. These results showed that of the nine anti-idiotype mAb produced only AIM.6 strongly inhibited binding of both radioiodinated and unlabeled H52. AIM.6 thus appeared to be truly paratope-specific and was chosen for further experiments.

Inhibition of Ab2 by purified LFA-1

A defining property of an internal image Ab2 (Ab2β) antibody is that the Ab2 blocks binding of Ab1 to antigen and that antigen blocks the binding of Ab2 to Ab1. In order to determine if AIM.6 anti-idiotype mAb was of the Ab2β type AIM.6 IgM was purified, radioiodinated and competed for binding to H52 against purified human LFA-1 and porcine LFA-1 which we have shown in a previous study to react with H52 (Hildreth *et al.*, 1989). Purified human CD45 was used as a negative control. Radioiodinated AIM.6 IgM (10 μCi/μg) at a concn of 0.25 μg/ml was competed against the purified proteins for binding to H52 in a solid phase assay as described in Materials and Methods. Binding of AIM.6 was not affected by CD45 but was inhibited by purified human LFA-1 and porcine LFA-1 in a dose-dependent fashion (Fig. 6) and at the highest concn of purified antigen (100 μg/ml) binding was inhibited 95 and 50% by human and porcine LFA-1, respectively. The lower degree of inhibition by poricine LFA-1 probably reflects the lower affinity of binding of the porcine antigen to H52 compared to human LFA-1 (Hildreth, unpublished). The relatively high concns of LFA-1 required to inhibit the binding of AIM.6 have also been noted when competing purified LFA-1 against labeled H52 suggesting a lower affinity of binding of H52 to soluble LFA-1 compared to cell surface antigen. Ab2 carrying the internal image of the Ab1 epitope can induce Ab3 with the same specificity as the corresponding Ab1

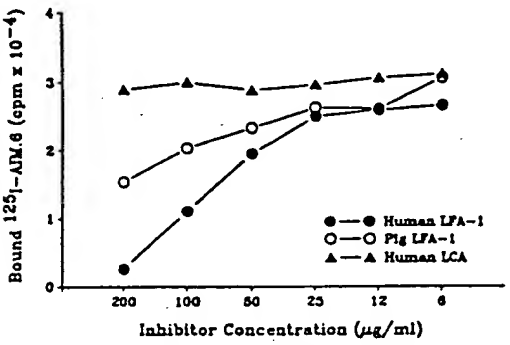

Fig. 6. Inhibition of AIM.6 binding to H52 by purified LFA-1. AIM.6 IgM was purified from ascites and radioiodinated as described in Materials and Methods. Human and porcine LFA-1 and human CD45 were purified from spleen and dilutions were prepared in PBS, 0.5% octyl glucoside. Diluted antigens (50 μl) were added to wells coated with H52 F(ab')$_2$ and after 45 min at 4°C an equal volume of ^{125}I-AIM.6 (50 ng) was added. Following a 1-hr incubation at 4°C the plate was washed five times.

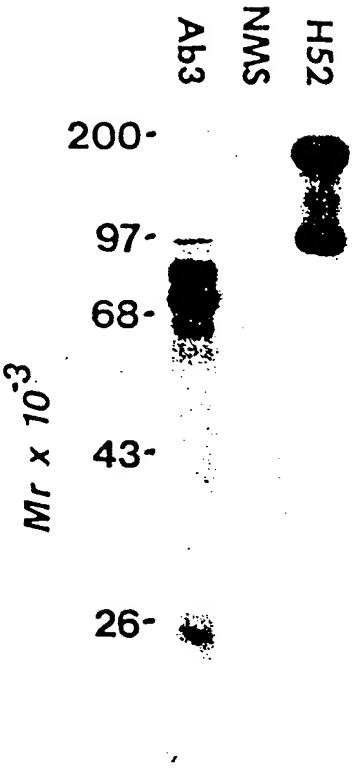

Fig. 7. Immunoprecipitation of LFA-1 by anti-AIM.6 Ab3 serum. HSB-2 cells were vectorially labeled with ^{125}I and immunoprecipitations were carried out as previously described (Hildreth and August, 1985). Pooled sera from normal (NMS) and AIM.6 IgM-immunized (Ab3) Balb/c mice were used as well as H52 IgG.

(Ab1'). Balb/c mice were immunized with purified AIM.6 to induce an Ab3 response. Sera from the immunized mice showed antibody binding to purified LFA-1 and to cells expressing LFA-1. The Ab3 sera also precipitated protein bands from labeled HSB-2 cells identical in mol. wt to those precipitated by H52 (Fig. 7). However, the sera precipitated much stronger bands of M_r 80,000, 72,000, 60,000, 30,000 and 25,000 (Fig. 7). The anti-AIM.6 Ab3 sera blocked binding of labeled AIM.6 but not H52 (data not shown) reflecting, as did the immunoprecipitation results, a low frequency of Ab1' antibodies among the Ab3 antibodies. The above results confirmed that the AIM.6 mAb carried an internal image of the CD18 epitope recognized by H52 and that AIM.6 met the criteria defined for β-type anti-idiotype mAbs.

Inhibition of cell–cell adhesion

Previous studies have demonstrated the involvement of LFA-1 and its ligands in the adhesion of a variety of cell types. In order to determine if AIM.6, which carries an internal image of the H52 CD18 epitope, recognized an adhesion ligand for LFA-1 we tested AIM.6 for inhibition of LFA-1-mediated cell–cell adhesion. For these studies we chose phorbol ester induced cell aggregation based on the results of previous studies showing that such aggregation can

be completely blocked by mAb against CD18 and ICAM-1 (Rothlein and Springer, 1986; Dustin and Springer, 1988). In these studies H52 IgG was used at a final concn of 2 μg/ml while AIM.6 and AIM.7 purified IgM were both used at a final concn of 100 μg/ml, about 10 times the H52 concn required for saturation binding with the number of cells used (Hildreth, unpublished). The mAb were first tested for inhibition of TPA-induced aggregation of PHA-stimulated T cell blasts. Whereas H52 and a mAb against the α-subunit of LFA-1 (MHM.24) completely inhibited aggregation of the blasts, neither AIM.6 nor the control anti-idiotype mAb had any effect (Table 1). The mAb were then tested against TPA-treated EBV-transformed B cells (SW.B). Again, phorbol ester-induced aggregation was completely inhibited by H52 and MHM.24 but was not affected by AIM.6 or AIM.7 (Table 1). TPA-induced aggregation of neutrophils was also inhibited by H52 and MHM.24, but much less efficiently than that of the other cell types, 61 and 47%, respectively. In addition to inducing cell–cell adhesion TPA also caused a substantial increase in neutrophil adherence to the culture wells. Neither neutrophil adherence to culture wells nor cell–cell adhesion were inhibited by AIM.6 or the control mAb (Table 1). The monocytic cell line U-937 has also been shown to undergo TPA-induced aggregation (Rothlein and Springer, 1986). U-937 cells showed no TPA-induced aggregation without pretreatment but aggregation of greater than 80% of the cells was obtained if the cells were first cultured for 2 days in the presence of TPA at 2 ng/ml. H52 and MHM.24 inhibited the TPA-induced aggregation of U-937 cells by greater than 90%. As with the other cell types tested there was no inhibition by either of the anti-idiotype mAb. By definition β-type Ab2 antibodies produced against Ab1 specific for the binding site of receptors should inhibit receptor binding if they recognize the ligand. To test the possibility that AIM.6 may have recognized a cell surface structure without inhibiting adhesion we tested it for binding in indirect assays to four different TPA-treated cell types. PHA blasts, U-937, a B cell line, and neutrophils, all of which undergo LFA-1-mediated adhesion. Results from all cells showed no significant binding of AIM.6 above that of the control antibody AIM.7. Furthermore, AIM.6 failed to immunoprecipitate any specific protein bands from radiolabeled PHA blasts (not shown).

Table 1. Effect of H52 and AIM.6 mAb on TPA-induced cell–cell adhesion[a]

mAb[c]	Cells[b]			
	PHA blasts	SW.B	Neutrophils	U-937
None	76[d]	91	87	81
AIM.6	75 (1.9)[e]	87 (4.4)	82 (5.8)	78 (3.8)
AIM.7	71 (9.1)	88 (4.2)	81 (6.0)	80 (1.3)
H52	0 (100)	0 (100)	34 (60.8)	3 (96.3)
MHM.24	0 (100)	2 (97.9)	46 (47.2)	5 (93.9)

[a]Cell adhesion assays were performed as described in Materials and Methods using cells at a density of 2×10^6/ml. Cells were pre-incubated with mAb before adding TPA to a final concn of 50 ng/ml followed by a 1-hr incubation at 37°C.

[b]PHA blasts were generated by culturing PBMC for 3 days in the presence of PHA at 0.5 μg/ml. Neutrophils were purified as described in Materials and Methods. U-937 cells were cultured for 2 days in the presence of TPA at 2 ng/ml before use in the adhesion assays. SW.B, an EBV-transformed B cell line, was used without pre-treatment.

[c]Antibody concns used were: H52 IgG, 2 μg/ml; AIM.6 IgM, 100 μg/ml; AIM.7 IgM, 100 μg/ml; MHM.24 ascites was used at a final dilution of 1:150.

[d]Per cent aggregation of cells was determined as described in Materials and Methods.

[e]Values shown in parentheses are per cent inhibition of aggregation by mAb.

Inhibition of T cell stimulation

Anti-CD18 mAb including H52 have been shown to inhibit a wide range of lymphocyte functions (Hildreth and August, 1985; Springer *et al.*, 1982) and more recently mAb against ICAM-1 have been shown to have similar effects, suggesting that interaction of LFA-1 with its ligand(s) is required for lymphocyte activation. To test whether AIM.6 recognized a ligand expressed transiently during lymphocyte activation we tested the antibody for inhibition of T cell activation by PHA. Normal PBMC were stimulated with increasing concns of PHA in the presence of purified AIM.6, AIM.7 and H52 Ig. At a concn of 2 μg/ml H52 completely inhibited stimulation at the lowest PHA concn (125 ng/ml) and inhibited 40% at the highest mitogen level (1000 ng/ml). In contrast, AIM.6 did not inhibit stimulation at any concn of PHA even though it was used at 50 times the concn of H52 (not shown).

Effect of temp on AIM.6 inhibition of H52 binding

We considered the possibility that the binding of AIM.6 to H52 and adhesion ligands may be temp-dependent since all competitive binding assays were carried out at 0°C whereas the functional studies were carried out at 37°C. We therefore compared the binding of AIM.6 to H52 at 0 and 37°C. AIM.6 bound equally well at 0 and 37°C to H52 in solid phase with a 50% maximal binding titer of 400 ng/ml (Fig. 8). However, in liquid phase immunoprecipitation experiments AIM.6 bound to H52 at 0 but not at 37°C (Fig. 8). AIM.6 was also tested for inhibition of H52 binding at 0 and 37°C. AIM.6 blocked binding of H52 at 0°C as shown above but did not inhibit binding at 37°C (data not shown). These results indicated that AIM.6 did not mimic the H52 CD18 epitope at 37° and bound to H52 with a very low affinity at this temp.

Frequency of the AIM.6 idiotype

If the β-subunit of LFA-1 (CD18) interacts directly with the adhesion ligand it is likely that a highly defined region or regions of CD18 mediate this interaction. This would imply that mAb against CD18 which inhibit ligand binding would likely share certain idiotopes. To determine whether the AIM.6 idiotype was conserved among anti-CD18 mAb a

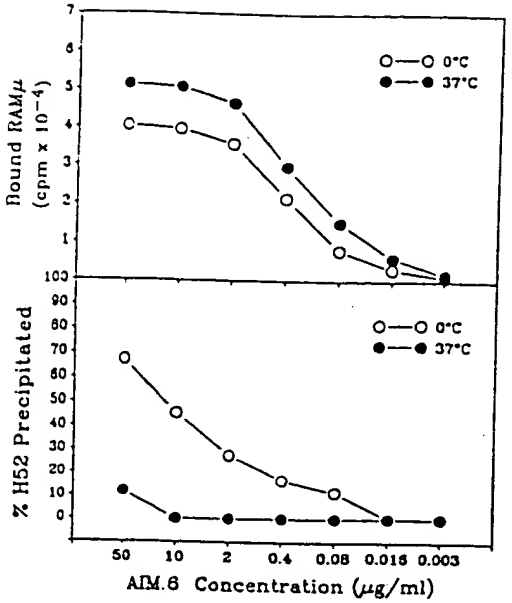

Fig. 8. Effect of temp on AIM.6 binding to H52. Upper panel: the binding of purified AIM.6 IgM to H52 $F(ab')_2$ was titrated in a SPRIA as described in Fig. 2. Identical assays were carried out in parallel at 0 and 37°C. Lower panel: purified AIM.6 IgM was titrated against ^{125}I-labeled H52 (50 ng) in parallel radioimmunoprecipitation assays carried out at 0 and 37°C. Immune complexes were brought down with RAMμ-coated fixed *Staphylococcus aureus*. Three per cent of the total input counts were precipitated in the absence of added AIM.6 IgM.

solid phase capture assay was used. A panel of 12 anti-CD18 mAb were captured onto the wells of PVC microtiter plates coated with RAM followed by addition of radiolabeled purified AIM.6 or RAMIg. Of the 12 anti-CD18 mAb tested only H52 and H5B9 were bound by AIM.6 (Fig. 9). Binding of the labeled RAMIg confirmed that similar amounts of each anti-CD18 mAb were captured onto the plate and a binding assay against HSB-2 target cells showed that the anti-CD18 mAb all had similar binding activities

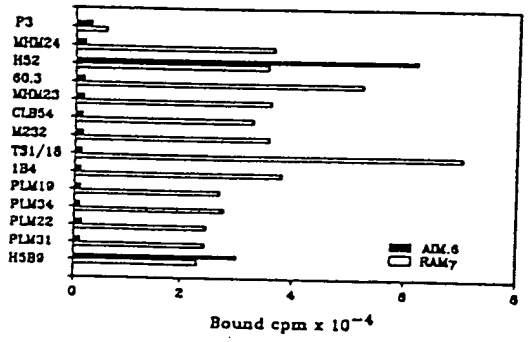

Fig. 9. Binding of AIM.6 to anti-CD18 mAb. mAb in the form of ascites fluids diluted 1:50 were captured onto duplicate wells coated with RAMγ. After washing away unbound mAb, ^{125}I-AIM.6 or ^{125}I-RAMIg was added to the wells followed by a 1-hr incubation. Culture supernatant from P3x653 cells was used as a negative control. All mAb used recognize epitopes on CD18 except MHM.24 which binds to the α-subunit of LFA-1 (CD11a).

(not shown). The results confirm those of a previous study in which it was shown that H52 and H5B9 recognize the same epitope on CD18 (Hildreth *et al.*, 1989). While the PLM series of anti-CD18 mAb used here do not inhibit leukocyte adhesion-related functions (Hildreth, unpublished) previous studies have demonstrated that 60.3, MHM.23, CLB54, M232, TS1/18 and IB4 all inhibit leukocyte adhesion (Patarroyo and Ansotegui, 1987). Thus the results show that not all inhibitory anti-CD18 mAb carry the AIM.6 idiotope.

DISCUSSION

The leukocyte adhesion receptor (LAR) family consists of three heterodimeric surface glycoproteins involved in a variety of leukocyte functions (Hildreth and August, 1985; Springer *et al.*, 1987). The LAR (LFA-1, Mac-1 and p150,95) are members of the large family of adhesion molecules known as integrins (Ruoslahti and Pierschbacher, 1987; Hynes, 1987). The ligands for most members of the integrin family are well characterized and a tripeptide sequence (RGD) in these ligands appears to be crucial for receptor binding (Ruoslahti and Pierschbacher, 1987). Mac-1 and p150,95 are known to bind to C3bi but the cell surface ligands for these receptors which mediate granulocyte intercellular adhesion have not been identified (Beller *et al.*, 1982; Hildreth and August, 1985; Sanchez-Madrid *et al.*, 1983*b*; Anderson *et al.*, 1986; Wright *et al.*, 1983; Patarroyo *et al.*, 1985*b*). ICAM-1, a member of the immunoglobulin supergene family, has been identified as one ligand of LFA-1 (Marlin and Springer, 1987; Rothlein *et al.*, 1986; Dustin *et al.*, 1986; Staunton *et al.*, 1988). Results of cell adhesion studies suggest that other structures expressed on leukocytes may also serve as LFA-1 ligands (Rothlein and Springer, 1986; Dustin and Springer, 1988). In the present study we have produced anti-idiotype antibodies against an anti-LAR mAb in an attempt to identify ligands of LFA-1. This approach has been successfully applied to other receptor–ligand systems (Monroe and Greene, 1986; Kauffman *et al.*, 1983; Chanh *et al.*, 1987; Hiernaux, 1988; Barel *et al.*, 1988).

Nine anti-idiotype mAb from three separate fusions were prepared against H52 and five of these Ab2 were found to recognize private idiotopes on H52. The other four Ab2 presumably recognize a framework determinant on IgG1 heavy chain or κ light chain. Three of the Ab2 recognizing private idiotopes on H52 inhibited the binding of H52 to some degree suggesting that they recognized a site on or close to the H52 paratope. One of the inhibitory Ab2 (AIM.6) was shown to carry an internal image of the H52 epitope on CD18. AIM.6 inhibited the binding of H52 to purified LFA-1 in solid phase and to cell surface expressed LFA-1. AIM.6 blocked the binding of unlabeled and radiolabeled H52 equally

well in contrast to the other inhibitory Ab2. The binding of AIM.6 to H52 was completely inhibited by purified human LFA-1 and porcine LFA-1 which is also recognized by H52. Mice immunized with purified AIM.6 produced Ab3 which bound to purified LFA-1 and which immunoprecipitated bands corresponding in mol. wt to those brought down by H52 but also precipitated other proteins. All these results clearly demonstrated that AIM.6 mimicked a CD18 epitope and was an Ab2β-type anti-idiotype mAb (Bona and Kohler, 1984).

The idiotype–anti-idiotype network theory predicts the recognition of receptor ligand by Ab2β produced against Ab1 specific for the receptor binding site. This hypothesis has now been proven in many experimental systems in which anti-idiotype antibodies have been used to identify receptors or ligands (Hiernaux, 1988; Monroe and Greene, 1986; Chanh *et al.*, 1987). The AIM.6 mAb was tested for recognition of an LFA-1 ligand in cell adhesion assays sensitive to blockade by H52 and other anti-LFA-1 mAb (Rothlein and Springer, 1986; Dustin and Springer, 1988; Patarroyo *et al.*, 1985*b*; Patarroyo *et al.*, 1986). Other studies have demonstrated that mAb against one of the ligands of LFA-1 (ICAM-1) inhibit adhesion in a system similar to the one used in this study (Rothlein and Springer, 1986; Dustin and Springer, 1988). Very high concns of the AIM.6 mAb did not inhibit cell adhesion in experiments in which much lower concns of H52 completely abolished cell–cell contact. To examine the possibility that certain ligands, of LFA-1 may be expressed only after inital cell–cell contact mediated by others receptors, AIM.6 was tested for inhibition of T cell activation. Mitogen stimulation of T cells which was quite sensitive to blockade by H52 was not affected by high concns of AIM.6. In other studies using IgM Ab2 against anti-receptor antibodies, inhibition of receptor–ligand interaction has been observed at concns substantially lower than those used for AIM.6 in the present study (Gramsch *et al.*, 1988). When tested at 37°C, AIM.6 failed to immunoprecipitate or inhibit the binding of H52. These results indicate that AIM.6 mAb mimics the H52 CD18 epitope at low temps but not at 37°C and may explain the low frequency of Ab1'-type Ab3 antibodies in the anti-idiotype response to AIM.6. This property of AIM.6 also precluded recognition of a LAR adhesion ligand in functional assays all of which are carried out at 37°C.

Although unlikely, AIM.6 may recognize a site on an LFA-1 ligand other than the LFA-1 binding site. If H52 recognizes a binding site on CD18 for adhesion ligands, then Ab2β produced against H52 should similarly recognize the binding site on the ligand. Results from our laboratory and from others show that there are multiple epitopes on CD18 recognized by inhibitory mAb (Hildreth *et al.*, 1989; Ware *et al.*, 1983). Consistent with this observation in the present study we found that only two of 12 anti-CD18 mAb bore the AIM.6 idiotope. The two anti-CD18 mAb recognized by AIM.6 have been previously shown to recognize the same epitope (Hildreth *et al.*, 1989). The association of multiple epitopes with inhibition of adhesion suggests that there may not be a single site on CD18 that serves as the binding site for ligands. However, if the binding site is an extended area on the exposed surface of CD18, then multiple epitopes could be associated with it. It is possible that H52 and other mAb against CD18 inhibit LFA-1-mediated adhesion by steric hindrance rather than recognition of a specific binding site. If the antibody does block by steric effects then it is not likely that Ab2β will recognize the ligand. Thus the success of the idiotype–anti-idiotype approach in identifying LAR ligands may depend on the choice of Ab1. For this reason we have begun to produce Ab2 against a panel of inhibitory anti-CD18 mAb that recognize different epitopes from that of H52.

The subunit of LFA-1 and the other LAR responsible for direct binding to the ligands has not been determined. The observation that LFA-1, Mac-1, and p150,95, which have different ligand specificites, all share the same β-subunit (Hildreth and August, 1985; Springer *et al.*, 1987; Sanchez-Madrid *et al.*, 1983*a*) strongly suggests that the α-subunits determine ligand specificity. Previous results from our laboratory showing that mAb against LAR α-subunits inhibit functions associated with the corresponding LAR whereas mAb against the common β-subunit inhibit all LAR functions (Hildreth and August, 1985) also support the idea that ligand specificity is determined by the α-subunits. All subunits of the LAR family have now been completely characterized and some clues related to ligand recognition can be drawn from the data. The α-subunits of Mac-1 and p150,95 are 63% identical at the amino acid level and this reflects their similarity at the functional level (Law *et al.*, 1987; Arnaout *et al.*, 1988; Corbi *et al.*, 1988; Corbi *et al.*, 1987). The LFA-1 α-subunit is only 35% identical to the α-subunits of Mac-1 and p150,95, consistent with the recognition of distinct ligands (Corbi *et al.*, 1988). The α-subunits of LAR which recognize cell surface bound ligands are only 25% identical to the α-subunits of integrins that recognize extracellular matrix proteins (Corbi *et al.*, 1988). The LAR α-subunits contain a stretch of 187 amino acids (the L-domain) for which there is no equivalent in the ECMR α-subunits (Corbi *et al.*, 1988; Arnaout *et al.*, 1988). The L-domain of LAR shows very significant homology to the A domains of von Willebrand Factor and to factor B, a component of the alternative complement pathway (Corbi *et al.*, 1988). The homology between the L-domain and factor B and the binding of factor B to C3b suggest that the L-domain of the α-subunits of Mac-1 and p150,95 may constitute the C3bi binding site. Comparisons of the sequences of the subunits of the LAR and ECMR integrins show that there is a significantly higher level of overall homology between the

β-subunits than between the α-subunits (Law *et al.*, 1987; Arnaout *et al.*, 1988; Corbi *et al.*, 1988; Corbi *et al.*, 1987; Kishimoto *et al.*, 1987). This would be expected if indeed the α-subunits in each subfamily determine ligand specificity while the β-subunits served a common function such as signal transduction or anchorage to cytoskeletal elements (Tamkun *et al.*, 1986; Horwitz *et al.*, 1986). There is, however, convincing evidence that the β-subunits play a direct role in formation of the ligand recognition site. In two separate studies utilizing chemical cross-linkers it has been shown that the β-subunits of placental vitronectin receptor and platelet IIb/IIIa are in direct association with bound ligand (Smith and Cheresh, 1988; D'Souza *et al.*, 1988). The above observations suggest both the α- and β-subunits play an important role in determining the specificity of integrins and for this reason we have also begun to generate Ab2 against mAb specific for inhibitory epitopes on the α-subunits of LAR.

Depending on the size and complexity of the binding site on LFA-1 for its ligands it may not be possible to produce an anti-idiotype antibody capable of mimicking the binding site. Antibodies specific for small molecules may use a pocket or groove for recognition and such binding sites appear to be easily mimicked by anti-idiotype antibodies (Kieber-Emmons and Kohler, 1986). In contrast, antibodies specific for an array of amino acids such as epitopes on the surface of a globular protein may have complex binding sites utilizing more extensive areas of the immunoglobulin surface (Kieber-Emmons and Kohler, 1986). In the latter case, although an Ab2 mAb may carry an image of a portion of the Ab1 epitope on the receptor, the area mimicked by the Ab2 may not be sufficient to result in mimicry of receptor function. However, Ab2β are known which mimic structures ranging from carbohydrates to catecholamines and, as stated above, Ab2β have already been produced which recognize protein receptors or ligands. Thus the success of the idiotype–anti-idiotype strategy for identifying LAR ligands would seem to depend on the nature of Ab1, primarily whether it inhibits adhesion by steric effects or by recognition of a specific receptor site, and on the successful generation of β-type Ab2. In this study we have demonstrated that the second requirement can be satisfied, having generated an Ab2 carrying the internal image of the LAR epitope recognized by H52. We are currently developing techniques that will allow distinction between anti-LAR mAb that block adhesion by steric hindrance and those that inhibit by recognition of specific binding sites.

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